

12
PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6: G01N 33/49, 33/48, 33/577, C12Q 1/68		A1	(11) International Publication Number: WO 99/15892 (43) International Publication Date: 1 April 1999 (01.04.99)
(21) International Application Number: PCT/AU98/00774		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 18 September 1998 (18.09.98)		Published <i>With international search report.</i>	
(30) Priority Data: PO 9319 22 September 1997 (22.09.97) AU			
(71) Applicant (<i>for all designated States except US</i>): FLINDERS TECHNOLOGIES PTY. LTD. [AU/AU]; Bedford Park, S.A. 5042 (AU).			
(72) Inventor; and			
(75) Inventor/Applicant (<i>for US only</i>): KALIONIS, Bill [AU/AU]; 13 George Road, Bellevue Heights, S.A. 5050 (AU).			
(74) Agents: SLATTERY, John, M. et al.; Davies Collison Cave, 1 Little Collins Street, Melbourne, VIC 3000 (AU).			

(54) Title: ENRICHMENT AND IDENTIFICATION OF TROPHOBlast CELLS IN MATERNAL PERIPHERAL BLOOD

(57) Abstract

A method for separation of trophoblast cells from a sample of peripheral blood of a pregnant mammal, particularly a pregnant human, comprises passing the peripheral blood sample through a selective filter to separate a fraction comprising trophoblast cells present in the peripheral blood sample.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		

- 1 -

ENRICHMENT AND IDENTIFICATION OF TROPHOBlast CELLS IN MATERNAL PERIPHERAL BLOOD.

5 This invention relates to a method for the isolation of fetal cells, and in particular trophoblast (placental) cells, from the peripheral blood of a pregnant mammal, especially a pregnant human. The isolation of these cells from maternal blood enables genetic and/or biochemical information about the fetus to be obtained, and also assists in the diagnosis of various conditions in pregnant women, such as
10 pregnancy induced hypertension.

The search for a non-invasive prenatal diagnostic test has led to the development of methods of extracting fetal and trophoblast cells from maternal blood. Monoclonal antibodies (Mabs) specific for trophoblast have been developed as part
15 of this research in a number of centres worldwide (Ganshirt D. et al, *Fetal and Maternal Medicine Review* (1995) 7:77). These are useful tools for detecting increased numbers of trophoblast in the peripheral blood of women.

Currently, prenatal testing is generally carried out on fetal cells obtained by either amniocentesis or chorionic villous sampling (CVS). Amniocentesis is normally performed around 16 weeks of gestation. The procedure involves attendance by skilled personnel to insert a needle into the amniotic sac of the fetus and remove between 20-30 ml of amniotic fluid. The amniotic fluid contains fetal cells to allow subsequent tests to be performed. This method of obtaining fetal cells is associated
20 with a risk of inducing a spontaneous abortion. In addition, if the subsequent genetic diagnosis of the fetus reveals an abnormality, the prospect of a mid-trimester pregnancy termination is both psychologically stressful and associated with some physical risk to the mother.

- 2 -

Chorionic villous sampling also requires the involvement of skilled personnel to take a small biopsy from the placenta of an 8-12 week old fetus. Again this procedure has some risk of inducing a spontaneous abortion, although the early diagnosis of any chromosomal abnormality makes the procedure more attractive than 5 amniocentesis. However, the need for skilled personnel and the possibility of inducing spontaneous abortion for both procedures means that current prenatal genetic assessments are made only on pregnant women who are deemed "at risk" of carrying a chromosomally defective fetus.

10 Trophoblast cells in the peripheral blood of pregnant women are derived from the placenta and are therefore fetal in origin. Recently, with the disclosure of antibodies, particularly monoclonal antibodies, reactive against human trophoblast specific epitopes, it has become feasible to isolate trophoblasts from the peripheral blood by use of immobilised trophoblast-specific antibody to bind to these 15 trophoblasts. International Patent Publication No. WO 90/06509 (Application No. PCT/AU89/00517), the disclosure of which is incorporated herein by reference, discloses a technique using these trophoblast-reactive monoclonal antibodies in an immunomagnetic separation system employing magnetically activated polystyrene beads, whereby trophoblast cells were isolated from maternal peripheral blood as 20 early as 8 weeks of gestation. (See also Mueller *et al.* *Histochemical J.* (1987) 19:288-296, Mueller *et al.* *Lancet* (1990) 336:197-200 and Jones *et al.* *Clin. Biochem. Revs.* (1994) 15:21-24, all of which are also incorporated herein by reference.)

25 Pregnancy induced hypertension (PIH) (or "pre-eclampsia") is thought to be fundamentally linked to a process involving deportation of trophoblast to the maternal circulation (Walker, J.J., *Br. J. Obstet. Gynaecol.* (1994) 101:639). The pathogenesis of PIH is believed to originate in the placenta. The condition may become systemic by embolisation of trophoblast cells into the maternal circulation. Trophoblast has been found at post-mortem in the lungs of eclamptics (Schmorl G. *Pathologische - 30 Anatomische Unter - suchungen über Puerperal eklampsie.* (1893) Vogel, Leipzig)

1-3-1

and in higher concentration in the uterine vein in pre-eclamptics (Chua, S. et al., *Br. J. Obstet. Gynaecol.* (1991) **98**: 973). It has been proposed that trophoblastic embolisation is the factor linking placental pathology with the systemic disorder in PIH circulation (Walker, J.J., *Br. J. Obstet. Gynaecol.* (1994) **101**:639). It has also been shown that trophoblast, both extravillous cytotrophoblast and multinucleate syncytiotrophoblast enters the peripheral circulation (Hawes, C.S. et al., *Am. J. Obstet. Gynecol.* (1994) **170**:1297). More recently, it has been shown that syncytiotrophoblast microvilli (STBM) are shed into the maternal circulation and are present in significantly increased amounts in pre-eclamptic women (Knight, M. et al., *Br. J. Obstet. Gynaecol.* (1998) **105**:632). Therefore, it is reasonable to expect that women with PIH will have elevated amounts of trophoblast in the peripheral blood.

The present invention is directed to a method for easily enriching and identifying trophoblast cells in maternal peripheral blood in the presence of a population of blood cell types. The enrichment, identification and analysis of trophoblast cells in peripheral blood provides a means by which non-invasive prenatal diagnosis can be carried out. This method is therefore of particular value in prenatal testing to obtain genetic and/or biochemical information about the fetus, and in diagnosis of conditions such as PIH

20

In accordance with this invention, there is provided a method for separation of trophoblast cells from a sample of peripheral blood of a pregnant mammal, particularly a pregnant human, which method comprises passing said peripheral blood sample through a selective filter to separate a fraction comprising trophoblast cells present in said peripheral blood sample.

Preferably, said selective filter is a filter having a pore-size of approximately 10 μm .

- 4 -

The fraction which is separated out from the peripheral blood sample by the selective filtration step of the present invention is a trophoblast-enriched fraction, which may then be further investigated, for example to specifically identify trophoblast cells in the fraction using a trophoblast-reactive antibody, such as a trophoblast-specific monoclonal antibody.

Thus, in one preferred embodiment, the present invention comprises selective filtration of a peripheral blood sample from a pregnant woman as broadly described above, followed on treatment of material retained on the filter with a monoclonal antibody specific for trophoblast cells (Hawes, C.S. et al., Proceedings of the 7th International Conference on Early Prenatal Diagnosis of Genetic Diseases, (1994) Jerusalem, pp 219) thereby enabling their detection.

Trophoblast cells have been found to be retained on 10 µm pore-size filters when blood from pregnant women is filtered. Filtration results in an enrichment of trophoblast cells, however other cells such as leukocytes are present and therefore it may be necessary to use trophoblast-reactive antibodies to unambiguously identify the trophoblast cells in a large population of other cell types. This enables the quantitation of trophoblast cells and the differentiation of normal women from those with PIH.

In a further aspect of this invention, there is provided a method for the determination of the level of trophoblast cells in a sample of peripheral blood of a pregnant mammal, particularly a pregnant human, which method comprises the steps of (i) separating a fraction comprising trophoblast cells from said peripheral blood sample by selective filtration, and (ii) identifying and quantifying the number of trophoblast cells in said fraction.

- 5 -

It will of course be appreciated that once the number of trophoblast cells in the blood sample has been quantified, this information can be used to identify pregnant women who are considered to be susceptible to, or predisposed to, PIH.

5. In a further embodiment of this invention, there is provided a kit for routine screening of pregnant women for levels of trophoblast in the peripheral blood, or specifically for women considered to be susceptible to, or predisposed to, PIH. In addition, the kit provides a means for testing and monitoring the effectiveness of antihypertensive drugs by measuring levels of trophoblast in the peripheral blood
- 10 prior to, during and after drug treatment. The kit comprises a selective filter for separation of a fraction comprising trophoblast cells from a peripheral blood sample, together with means for identifying trophoblast cells in the fraction, such as a labelled trophoblast-reactive antibody, (particularly a trophoblast-reactive monoclonal antibody) or a probe specific for trophoblast mRNA.

15

Trophoblast cells in the fraction enriched by filtration can be identified by the process of *in situ* hybridization. *In situ* hybridization is a well established method for locating nucleic acid sequences (RNA or DNA) in the cytoplasm or nucleus of a cell. Genetic probes to detect specific messenger ribonucleic acid (mRNA) molecules in the cytoplasm of trophoblast cells can be used to identify these cells. These genetic probes have the property that they do not significantly hybridize to mRNA molecules in cells in peripheral maternal blood other than trophoblast cells. In addition, genetic probes for human chromosomes are available that are suitable for *in situ* hybridization and allow the genetic analysis of trophoblast cells.

20

By carrying out *in situ* hybridization with probes specific for trophoblast mRNA and with probes specific for human chromosomes in the nucleus of the trophoblast cell, it is possible to obtain information on the chromosomal complement of the fetus and thereby carry out prenatal diagnosis.

25

- 6 -

Thus, in a further aspect, the present invention provides a method for obtaining fetal genetic and/or biochemical information from trophoblast cells, which method comprises the steps of (i) separating a fraction comprising trophoblast cells from a sample of peripheral blood of a pregnant mammal, particularly a pregnant human, by selective filtration, and (ii) obtaining genetic and/or biochemical information from trophoblast cells in said fraction.

In this aspect, the present invention also provides a kit for carrying out non-invasive prenatal diagnostic tests on maternal blood samples. Such a kit comprises a selective filter for separation of a fraction comprising trophoblast cells from a peripheral blood sample, optionally means such as a labelled trophoblast-reactive antibody, for example a labelled monoclonal antibody, and/or a probe specific for trophoblast mRNA for identifying trophoblast cells, and means for obtaining genetic and/or biochemical information from trophoblast cells.

15

Methods of *in situ* hybridization using genetic probes for biochemical and genetic analysis are well known in the art and are described, by way of example, in International Patent Publication No. WO 97/263241 (Application No. PCT/AU97/00020), the disclosure of which is incorporated herein by reference.

20

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

25

Detailed Description of the Invention

(1) Blood Collection

Typically, blood samples from pregnant women are collected into silicon-coated, ethylenediamine tetraacetic acid (EDTA)-treated tubes to

- 7 -

prevent coagulation. Blood sample volume can be in the range of 5-100ml, preferably 20ml.

(2) Blood Treatment

5 The blood may be filtered directly without any further treatment. Alternatively, the blood can be diluted with a isotonic buffer to reduce the viscosity prior to filtering. The blood can be treated using a variety of techniques known to those with skill in the art that will lead to the lysis of erythrocytes by employing hypotonic buffers. These methods can reduce the numbers of cells that need 10 to be filtered and also reduce the incidence of coagulation.

(3) Blood Filtration

Blood is filtered through a 10 µm pore-size filter, held in a filtration device. Typically, a suitable device consists of an upper reservoir to hold the blood 15 sample, and a leak-proof device attached to the upper reservoir to hold the filter that allows material to pass through the filter into a collecting reservoir. Prior to filtration, a solution of ethanol/ethylsulphate/ isopropyl alcohol/siloxane may be passed through the filtration device and filter, followed by rinsing with PBS. This treatment reduces non-specific adhering of cells to the filtration 20 device and filter.

Cells retained on the filter and the upper reservoir walls are washed with an isotonic buffer to ensure that all cells that can pass through the filter, come in contact with the filter.

25

(4) Treatment of retained cells

The retained cells are washed back off the filter with isotonic buffer (such as PBS), centrifuged at 100g to 4000g, preferably 400g, at 0°-25°C, preferably 4°C, for 1-60 min, preferably 15 minutes. The cells are resuspended in the

for analysis and dilution

- 8 -

buffer to a volume of 5-500 μ l, preferably 100 μ l, and pipetted onto a solid support such as a glass microscope slide in 2-200 μ l, preferably 20 μ l, aliquots.

- 5 (5) Addition of trophoblast-reactive antibodies for measuring trophoblast in the peripheral blood.

Trophoblast-reactive antibodies are applied to cells on the slide. Trophoblast antibodies useful in this invention are those that react with trophoblast cell types and do not react significantly with other cell types found in the blood of pregnant women. The antibodies may be polyclonal or monoclonal antibodies.

10 Trophoblast-reactive antibodies include, but are not restricted to, monoclonal antibodies (Mabs) FDO66Q, FDO338P, FDO161G, FDO46B and FDO202N (Flinders Technologies Pty. Ltd, Flinders University of South Australia, Bedford Park, South Australia). Antibodies can be used individually or as mixture of several antibodies. Antibodies are used at dilutions ranging from undiluted to

15 1 in 10,000, preferably 1 in 4, in PBS for the antibodies referenced above.

Antibodies are incubated with the cells for a period of 30 min to 24 hours, preferably 16 hours, at 4°-28°C, preferably 4°C.

20 Hybridoma cell lines expressing Mabs FDO66Q, FDO338P and FDO161G were deposited at the American Type Culture Collection, Rockville, Maryland, U.S.A. on March 10, 1994, under ATCC Nos. HB11569, HB11570 and HB11571, respectively.

- 25 (6) Detection and quantitation of trophoblasts in the peripheral blood.

Cells on the slides are incubated in 5% normal goat serum for 5 min to 24 hours, preferably 40 min, at 4°-28°C, preferably 20°C, to block non-specific binding of the secondary antibody. Other blocking agents can be used. A labelled secondary antibody is applied to the cells at a dilution of 1:2 to 1:

- 9 -

5000, preferably 1:400, and incubated for 5 min to 16 hours, preferably 60 min, at 4°-28°C, preferably 20°C.

In a preferred embodiment of this invention, the secondary antibody is labelled
5 with a fluorochrome such as 7'-amino-4-methylcoumarin-3'-acetic acid (AMCA), Coumarin, Fluorescein, R-phycoerythrin, Rhodamine, Texas red, Ultralight, cyanin 3 (Cy-3) or cyanin 5 (Cy-5). Cells are then detected with epifluorescence or confocal microscopy with the appropriate filter for the particular fluorochrome. The secondary antibodies can be detected by other
10 means such as enzyme-mediated reporter systems. In these systems, the secondary antibody is labelled with an enzyme such as horse radish peroxidase or alkaline phosphatase. Such enzyme mediated reporter systems catalyse the precipitation of a visible product at the site of binding of the secondary antibody and the cells are detected by light microscopy. The secondary antibody can also be labelled with metals such as gold or ferritin
15 and the cells are detected by reflection contrast microscopy. The secondary antibody can be labelled with a radioactive isotope such as I¹²⁵ and detected by autoradiography.

20 In another embodiment of this invention, the primary antibody or antibodies are labelled with the reporter systems described above, eliminating the requirement for a secondary antibody.

(7) Non-invasive prenatal diagnostic tests on maternal blood samples by
25 detection of trophoblast-specific messenger RNA and analysis of the chromosomal content of trophoblast cells using *in situ* hybridization.

30 Hybridization is a technique that is based on allowing polynucleotide strands to form double-helical segments through hydrogen bonding between complementary base pairs. The polynucleotide strands can be those of

- 10 -

single-stranded nucleic acids or can be derived from double-stranded nucleic acids. *In situ* hybridization is a method for determining the localization of specific nucleic acids within a cell and involves incubating cells with a labelled genetic probe to allow hybridization to occur and then visualizing the hybridized probe by detection of the label.

- 5 (a) Genetic probes for detecting trophoblast-specific mRNA and identifying specific human chromosomes.

A preferred agent for detecting an intracellular nucleic acid is a genetic probe.
10 A genetic probe is a substance that is used to identify a gene or a gene product and can be of the form of genetic material such as DNA (deoxyribonucleic acid), RNA (ribonucleic acid) or synthetic oligonucleotides. Genetic probes may contain natural or chemical derivatives of the normal components of nucleic acids that include guanine, adenine, uridine, thymidine, or cytosine.
15

Several genes express mRNA in trophoblast cells but do not significantly express mRNA in other cell types found in peripheral maternal blood. These genes include, but are not limited to, 3 β -hydroxysteroid dehydrogenase
20 (3 β HSD) (Hawes, C.S. et al., (1994) *J. of Molec. Endocrinology* 12:273), the human placental lactogen hormone gene (*hPL*) (Latham, S.E. et al., *Prenatal Diagnosis*, submitted), the homeobox genes *Dlx-8*, *Mox2A*, *Msx-2* and *HB24* and the *SP1* gene. Genes from which genetic probes can be derived that are useful for this invention, are those that express mRNA in trophoblast cells but are not significantly expressed in other cell types in the peripheral blood of pregnant women.
25

30 Genetic probes for specific human chromosomes that are suitable for *in situ* hybridization and allow the genetic analysis of trophoblast cells are known to those with skill in the art (For examples see Zheng, Y-L. et al., (1995) *Prenatal*

- 11 -

Diagnosis 15:897; Simpson, J.L. et al., (1995) *Prenatal Diagnosis* 15:907; Johansen M. et al., (1995) *Prenatal Diagnosis* 15:921).

(b) Types of probes

5 Nucleic acid probes may take the form of cloned nucleic acids, where the DNA fragment of interest is inserted into a vector and amplified inside appropriate host cells. The amplified DNA is extracted and purified. Common vectors include bacterial plasmids, bacterial viruses, yeast artificial chromosomes and cosmids. Synthetic oligonucleotides, 15 to 50 base pairs, in length may also be used and are prepared using a DNA synthesizer. DNA probes can also be prepared by amplification using the polymerase chain reaction, a process that relies on the use of suitable oligonucleotide primers that flank the DNA to be used as a probe. Probes can be double-stranded probes such as double-stranded DNA or complementary DNA (cDNA) or single-stranded 10 probes such as single-stranded DNA or RNA or oligonucleotides .

15

(c) Preparation of probes

Probes may be made detectable using labels. Labels can be incorporated into probes by enzymatic or chemical means. Double-stranded probes can be labelled by using a variety of DNA polymerases using known methods such as random-primed DNA labelling, nick translation, or labelling with Taq DNA polymerase in the polymerase chain reaction. Single-stranded RNA probes can be prepared from plasmid vectors by *in vitro* transcription using RNA polymerases such as T3, T7 and SP6. Viral vectors such as M13 can also be 20 used to prepare single-stranded DNA. Oligonucleotides can be labelled by end-labelling or tailing. Nucleic acid probes can be labelled enzymatically with a variety of labels. These include but are not limited to nucleotide derivatives of digoxigenin, biotin and fluorochromes. In addition, nucleic acid probes can be labelled chemically with a variety of labels including photodigoxigenin, 25 photobiotin, 2-acetylaminofluorene, sulphone groups, mercury, fluorochromes, 30

- 12 -

dinitrophenol, and psoralen. Other labels include but are not limited to enzyme substrates, enzyme inhibitors, coenzymes, chemiluminescers and bioluminescers. Methods to prepare labelled nucleic acid probes are known to those with skill in the art (see Leitch, A.R. et al., (1994) in *In Situ Hybridization*, Bios Scientific, Oxford, England; Hames, B.D. and Higgins, S.J., (1988) in *Nucleic Acid Hybridization: a practical approach*, IRL Press, Oxford, England).

The above methods involve the labelling of the genetic probe prior to hybridization with the target. However the genetic probe can be labelled following hybridization of the probe to the target using the technique of primed in situ labelling (PRINS) (Koch, J. et al., (1991) *Genet. Anal. Techniques Applications* 8:171). DNA probes in the form of oligonucleotides, PCR products or DNA fragments are hybridized to the target nucleic acid. The hybridized DNA then acts as a primer for the incorporation of labelled nucleotides in situ. For labelling of DNA targets, DNA polymerase is used. For RNA targets, reverse transcriptase is used to synthesize nucleic acid along the RNA template. Labels that can be incorporated into nucleic acid probes by enzymatic means have been described above.

In a preferred embodiment of this invention, the probe is labelled with a fluorochrome-nucleotide. Probes for the identification of trophoblast-specific mRNA have incorporated into them a different fluorochrome-nucleotide than that incorporated into the probe, or probes, used for the detection of chromosomes in the nucleus. The use of different fluorochromes for the trophoblast-specific mRNA probe and the chromosome probe allows simultaneous detection of the probes in the same cell by use of an appropriate emission wavelength filter and epifluorescence microscopy.

30 (d) Genetic probe size and concentration

- 13 -

The probe must be small enough to diffuse into the dense matrix of the cell whilst also being of sufficient length to maximise the hybridisation rate. Nucleic acid probes suitable for this invention are 100bp-1000bp, preferably 200bp-400bp, in length. Oligonucleotide probes suitable for this invention are 5 15bp-50bp in size. Parameters that affect probe size and concentration for *in situ* hybridization are known to those with skill in the art (Leitch, A.R. et al., (1994) in *In Situ Hybridization*, Bios Scientific, Oxford, England; Hames, B.D. and Higgins, S.J. (1988) in *Nucleic Acid Hybridization: a practical approach*, IRL Press, Oxford, England).

10

(e) Fixing Cells

Blood collection, blood treatment and blood filtration are as described above. The retained cells are washed back off the filter with isotonic buffer such as PBS, centrifuged at 100-4000g, preferably 400g, at 0°-25°C, preferably 4°C, 15 for 1-60 min, preferably 15 minutes. The cells are resuspended in a fixative to preserve cell morphology and prevent diffusion of intracellular nucleic acids from the cell during subsequent treatments. A fixative suitable for this invention is 0.4%-10% (w/v), preferably 4% (w/v), paraformaldehyde in PBS. Other fixatives suitable for the process of *in situ* hybridization are known to those with skill in the art (Leitch, A.R. et al., (1994) in *In Situ Hybridization*, Bios Scientific, Oxford, England; Hames, B.D. and Higgins, S.J. (1988) in *Nucleic Acid Hybridization: a practical approach*, IRL Press, Oxford, England).

20

(f) Permeabilizing Cells

25

Cells may be permeabilized using a permeabilizing agent. A permeabilizing agent is any compound which facilitates access of the hybridization probe to the cytoplasm of the cell. Permeabilization is an optional treatment and may not be necessary in some cases. A suitable permeabilizing agent for this invention is Proteinase K. Cells are permeabilized by treatment with 30 Proteinase K in PBS (PBS=0.15M NaCl, 10mM Na-phosphate; pH 7.2) buffer

- 14 -

at a concentration of 10-500 µg/ml, preferably 100 µg/ml, for 1-120 min, preferably 10 min at 18°-42°C, preferably room temperature. Permeabilization is stopped by replacing the Proteinase K solution with 0.02%-2% (w/v), preferably 0.2% (w/v), glycine in PBS for 1-20 min, preferably 2 min, at 18°-42°C, preferably room temperature. Stopping the reaction with glycine is an optional treatment and may not be necessary in some cases. Other permeabilizing agents suitable for the process of *in situ* hybridization are known to those with skill in the art (Leitch, A.R. et al., (1994) in *In Situ Hybridization*, Bios Scientific, Oxford, England; Hames, B.D. and Higgins, S.J. (1988) in *Nucleic Acid Hybridization: a practical approach*, IRL Press, Oxford, England).

5 (g) Post-Fixing Cells

Once permeabilized, fixation of the cells may be repeated. The fixatives and methods previously described above may be used for post-fixation of cells. Post-fixation is optional and may not be necessary in all cases. Removal of the fixative can be accomplished by a series of washes for 2-20 min each, preferably 5 min each, in PBS.

10 (h) Prehybridization

Prehybridization is an optional treatment used to minimise hybridisation of the probe to non-specific target molecules. Cells are resuspended in a prehybridization solution containing, for example, 50% formamide, 2x SSC (Ix SSC = 0.15M NaCl, 0.015M sodium citrate), 10% dextran sulphate and blocking DNA (or RNA) at 1 mg/ml. Blocking DNA (or RNA) is non-specific DNA (or RNA) that does not hybridize with the probe and reduces non-specific hybridization by binding to molecules in the cytoplasm or nucleus that would otherwise bind probe or detection reagents. Other hybridization solutions suitable for the process of *in situ* hybridization are known to those with skill in the art (Leitch, A.R. et al., (1994) in *In Situ Hybridization*, Bios Scientific,

- 15 -

Oxford, England; Hames, B.D. and Higgins, S.J. (1988) in *Nucleic Acid Hybridization: a practical approach*, IRL Press, Oxford, England). Prehybridization is carried out at 30°-50°C, preferably 37°C for 30 min to 16 hours, preferably 2 hours, to block non-specific binding of the probe. The temperature and duration of the prehybridization treatment are varied depending on the cell type and probe used. Following prehybridization, the excess prehybridization solution is removed.

(i) Probe Denaturation

Prior to hybridization, all double stranded nucleic acids must be denatured. Single-stranded nucleic acid probes and mRNA in the cytoplasm do not require denaturation. Denaturation is essential for double-stranded nucleic acid probes and for chromosomal DNA in the nucleus of the cell. Denaturation of the double-stranded nucleic acid probes and chromosomal DNA may be accomplished separately prior to hybridization. Methods for denaturing double stranded DNA include but are not restricted to, alkali or acid treatment, heat and organic solvents (Leitch, A. R. et al.(1994) in *In Situ Hybridization*, Bios Scientific, Oxford, England; Hames, B.D. and Higgins, S.J. (1988) in *Nucleic Acid Hybridization: a practical approach*, IRL Press, Oxford, England). The preferred method for denaturation in this invention is combined denaturation of the double-stranded nucleic acid probes and chromosomal DNA in the presence of a hybridization buffer that contains a chaotropic agent such as formamide (see below). Denaturation occurs at approximately 30°C above the T_m (see below)

(j) Hybridization

Hybridisation depends on the ability of denatured nucleic acids to reanneal with complementary strands in a hybridisation solution maintained at a temperature that is just below their melting point. The T_m is defined as the temperature at which half the nucleic acids are present in single-stranded

- 16 -

form. The Tm and reannealing of the nucleic acids is affected by temperature, pH, concentration of monovalent cations and the presence of organic solvents. The broad maximum rate for nucleic acid reannealing occurs from 10°-30°C below the Tm. For complementary nucleic acid sequences hybridisations usually are carried out at 65°-68°C in aqueous solution and 30° -55°C in formamide containing solutions.

A hybridization solution suitable for this invention contains 30%-60%, preferably 50%, formamide, 0.1x-SSC-6xSSC, preferably 2xSSC, 5%-10% (w/v), preferably 10% (w/v), dextran sulphate, 100 µg-5 mg/ml, preferably 1 µg/ml, fish sperm blocking DNA, 1-10 ng/µl, preferably 5 ng/µl, labelled trophoblast mRNA specific probe and 1-10 ng/µl, preferably 5 ng/µl, labelled human chromosome specific probe. The hybridization solution is added to the cells on the glass slide, a coverslip is applied and the edges sealed with rubber cement, and then the slide is heated to 70°-90°C, preferably 75°C, for 2-20 min, preferably 10 min, to denature the probes and chromosomal DNA in the nucleus. Hybridization is allowed to occur for 30 min to 48 hours, preferably 4 to 16 hours, at 30°-55°C, preferably 37°C.

Probes can be hybridized simultaneously or in succession.

Other hybridization solutions suitable for *in situ* hybridization can be used and are known to those with skill in the art (Leitch, A.R. et al., (1994) in *In Situ Hybridization*, Bios Scientific, Oxford, England; Hames, B.D. and Higgins, S.J. (1988) in *Nucleic Acid Hybridization: a practical approach*, IRL Press, Oxford, England). In general, hybridization solutions will contain a chaotropic agent such as formamide that decreases the Tm of nucleic acid hybrids and allows hybridizations to be performed at lower temperatures. This is desirable since cell morphology is adversely affected when cells are exposed to high temperatures over long periods of time. The presence of formamide also

- 17 -

allows the denaturation of probes by heating to approximately 30°C above the Tm. The hybridization solution also contains monovalent cations to stabilize the hybrids once formed, a buffer, and blocking DNA (or RNA). The hybridization solution may also contain dextran sulphate or other polymers such as polyethylene glycol and non-polymers such as phenol to increase the hybridisation rate, detergents such as sodium dodecyl to act as wetting agents and as permeabilizing agents to assist in probe penetration into the cytoplasm, EDTA or citrate to remove divalent cations and bovine serum albumin (BSA) or Denhardt's reagent (0.02% Ficoll, 0.02% polyvinyl pyrrolidone and 0.02% BSA) can also be included to reduce non-specific hybridization.

10 (k) Removing non-specifically bound probe

Hybridization solution is removed and the cell suspension is washed to remove non-specifically bound probe. The wash buffer, washing time, temperature and frequency of washes can vary depending on the probe used. Removal of non-specifically bound probe usually carried out at about 15°-20°C below the Tm. A suitable washing buffer for this invention is 0.01xSSC-6xSSC, preferably 2xSSC, at 30°-45°C, preferably 37°C. Other wash buffers suitable for *in situ* hybridization are known to those with skill in the art (Leitch, A.R. et al., (1994) in *In Situ Hybridization*, Bios Scientific, Oxford, England; Hames, B.D. and Higgins, S.J. (1988) in *Nucleic Acid Hybridization: a practical approach*, IRL Press, Oxford, England).

15 (l) Detection and visualization of the specifically bound probe

20 The methods for detection and visualization of the bound probe will depend on the type of label incorporated into the probe. In a preferred embodiment of this invention, the label is a fluorochrome-nucleotide that can be detected directly since it is incorporated into the nucleic acid probe by enzymatic labelling. The signal from each probe is visualized directly using

- 18 -

epifluorescence microscopy employing a wavelength emission filter appropriate for the particular fluorochrome to be detected.

In another embodiment of this invention, the fluorochrome is conjugated to a primary antibody that has been raised to the label incorporated into the nucleic acid probe. The fluorochrome-conjugated antibody can also be used as a secondary antibody and is raised to the primary antibody that is used to detect the label incorporated into the probe.

Other detection and visualization systems suitable for *in situ* hybridization are known to those with skill in the art (Leitch, A.R. *et al.*, (1994) in *In Situ Hybridization*, Bios Scientific, Oxford, England; Hames, B.D. and Higgins, S.J. (1988) in *Nucleic Acid Hybridization: a practical approach*, IRL Press, Oxford, England).

(8) In another embodiment of this invention, *in situ* hybridization is carried out following the detection of trophoblast cells using trophoblast-reactive antibodies. Methods for fixation of cells, following antibody detection, that allow subsequent *in situ* hybridization to be carried out are known to those with skill in the art (Zheng, Y-L. *et al.*, (1995) *Prenatal Diagnosis* 15:897).

(9) A kit for measuring trophoblast levels in blood may comprise 10 μ m filters, a solution of ethanol/ethylsulphate/isopropyl alcohol/siloxane and optionally a device to hold the filter in place that allows material to pass through the filter and the collection of retained cells on the filter for analysis. In addition, the kit comprises either:

(a) unlabelled antibodies reactive to trophoblast, but not significantly reactive to other cell types in blood such as unlabelled monoclonal antibodies FDO46B, FDO161G and FDO202N and

- 19 -

- (b) labelled secondary antibodies and necessary reagents for their detection;
or
labelled antibodies reactive to trophoblast, but not significantly reactive to other cell types in blood such as labelled monoclonal antibodies FDO46B,
5 FDO161G and FDO202N, for detecting trophoblast-Mab complexes directly, or by means of detection reagents which may be included in the kit, eg enzyme substrates.
- (10) A kit for carrying out non-invasive prenatal diagnostic tests on internal blood samples may comprise 10µm filters, a solution of ethanol/ethylsulphate/isopropyl alcohol/siloxane and optionally a device to hold the filter in place that allows material to pass through the filter and the collection of retained cells on the filter for analysis;
and
15 (a) a fixative
(b) a permeabilizing agent
(c) a hybridization solution
(d) a labelled hybridization probe, or probes, for detecting trophoblast-specific mRNA but that does not significantly detect mRNA of other cell types
20 (e) a labelled hybridization probe, or probes, for detecting a specific human chromosome or chromosomes
(f) necessary reagents for the detection of the label on each of the probes
- 25 Further features of the present invention are more fully described in the following Example(s). It is to be understood, however, that this detailed description is included solely for the purposes of exemplifying the present invention, and should not be understood in any way as a restriction on the broad description of the invention as set out above.

- 20 -

EXAMPLES

Example 1: Measurement of trophoblast levels in blood.

A 20 ml peripheral venous blood sample was collected from six women during pregnancy. Three women had a diagnosis of PIH (two had proteinuric PIH), as defined by widely accepted standards (Davey, D.A. and MacGillivray, I. *Clinics of Experimental Hypertension-Hypertension in Pregnancy* (1986) B5 (1): 97), and three women of similar parity and gestation (+/- 2 weeks) with normal pregnancies served as controls. Two volunteers gave blood samples as non-pregnant controls. Term placenta dispersed in PBS, was pipetted onto glass slides and dried in air at room temperature was used as a positive and negative control for the Mabs.

Prior to filtration, a 20 ml solution of ethanol/ethylsulphate/isopropyl alcohol/siloxane is passed through the filtration device and filter, followed by rinsing with PBS.

All blood samples were collected into silicon-coated, EDTA tubes and then filtered through 10 µm pore-size filters (Pall Corporation, UK). The retained cells were washed back off the filter with PBS, centrifuged at 400g, at 4°C, for 15 minutes down to a volume of approximately 100 µl and pipetted onto glass slides in 20 µl aliquots, allowed to dry in air at room temperature overnight and incubated with Mabs FDO46B, FDO161G and FDO202N (Flinders Technologies Pty. Ltd, Flinders University of South Australia, Bedford Park, South Australia) diluted 1:4 in PBS, overnight at 4°C. The slides were then incubated in 5% normal goat serum (Jackson ImmunoResearch, Penn, USA) for 40 minutes and then in a 1:400 dilution of Cy3 conjugated F(ab')₂ affinipure goat-anti-mouse IgG (H+L) (min x Hu, Bov, Hrs Sr Prot) (Jackson ImmunoResearch, Penn, USA) antibody for one hour at room temperature. Bound antibody was viewed immediately using fluorescence microscopy with a Rhodamine filter and the trophoblast cells counted to give a count per 20mls of blood. Table 1 shows significantly increased numbers of trophoblast-antibody-reactive cells

- 21 -

in women with PIH compared to matched controls that do not show PIH. Table 1 also shows that women without PIH also contain trophoblast antibody-reactive cells.

TABLE 1 Trophoblast cells in the peripheral blood per 20ml) showing increased numbers of trophoblast cells in women with PIH compared to matched controls that do not show PIH.

	Age (years)	Parity	Gestation	Diagnosis	Trophoblast Count (per 20 ml)
Patient 1	26	0	32	Proteinuric PIH	59
Patient 2	26	0	30	Proteinuric PIH	37
Patient 3	36	1	35	PIH	82
Control 1	24	0	33	Normal	7
Control 2	27	0	32	Normal	5
Control 3	32	1	37	Normal	8

Example 2: Non-invasive prenatal diagnostic tests on maternal blood samples.

A cDNA fragment encoding the 3β HSD gene is excised from a plasmid cDNA clone pCMVSH3 β -HSD (Lorenz et al, (1990) *Endocrinology* 126:2493) by restriction digestion with EcoRI/BamHI. The DNA fragment is labelled with tetramethyl-rhodamine-6-dUTP using the random-primed labelling kit from Boehringer Mannheim (DIG-High Prime kit, 1995 Catalogue No. 1-585-606) and following the manufacturer's instructions included in the kit. A human satellite DNA probe specific for human chromosome Y, that is fluorescein-12-dUTP labelled, is obtained from Boehringer Mannheim (1995 Catalogue No. 1-558-692).

- 22 -

Prior to filtration, a 20 ml solution of ethanol/ethylsulphate/isopropyl alcohol/siloxane is passed through the filtration device and filter, followed by rinsing with PBS.

5 A 20 ml peripheral venous blood sample is collected from women during pregnancy. All blood samples are collected into silicon-coated, EDTA tubes and then filtered through 10 µm pore-size filters (Pall Corporation, UK). The retained cells are washed back off the filter with PBS, centrifuged at 400g, at 4°C, for 15 minutes down to a volume of approximately 100 µl and pipetted onto glass slides in 20 µl aliquots,
10 and then allowed to dry in air at room temperature overnight.

Cells on the slide are fixed by addition of 4% paraformaldehyde in PBS (PBS=0.15M NaCl, 10mM Naphosphate; pH7.2) and then washed twice for 5 min in PBS. Cells are permeabilized by treatment with Proteinase K at 100 µg/ml for 10 min
15 at room temperature. Permeabilization is stopped by replacing the fixing solution with 0.2% (w/v) glycine in PBS for 2 min at room temperature. The cells are then postfixed in paraformaldehyde as described above. The slides are washed twice for 5 min in PBS.

20 Hybridization solution contains 50% formamide, 2xSSC, 10% (w/v) dextran sulphate, fish sperm DNA for blocking at 1mg/ml, 5ng/µl tetramethyl-rhodamine-6-dUTP labelled 3 β HSD probe, 5ng/µl fluorescein-12dUTP labelled human chromosome Y probe. The hybridization probe solution is added to the cells, a glass cover slip is placed on top of the cells and the coverslip is sealed
25 at the edges with rubber cement. The slides were heated to 72°C for 10 min to denature the probes and chromosomal DNA in the nucleus. Hybridization is for 16 hours at 37°C. After hybridization, the cover slip is removed. Non-specifically hybridized probe is removed by washing the slides 3 times for 5 min in 2xSSC at 37°C.

- 23 -

The signal from each probe is visualised directly using epifluorescence microscopy. The trophoblast specific mRNA hybridization signal from the rhodamine-6-dUTP labelled $3\beta HSD$ probe is detected with a 615nm emission wavelength filter. The hybridization signal from the fluorescein-12-dUTP labelled 5 human chromosome Y probe is detected with a 523nm emission wavelength filter. Only those cells that are positive for both the trophoblast specific mRNA hybridization signal and the human chromosome specific probe are used for analysing the chromosome content of the fetus.

10

- 24 -

CLAIMS:

1. A method for separation of trophoblast cells from a sample of peripheral blood of a pregnant mammal, which method comprises passing said peripheral blood sample through a selective filter to separate a fraction comprising trophoblast cells present in said peripheral blood sample.
2. A method according to claim 1, wherein the pregnant mammal is a pregnant human.
3. A method according to claim 1, wherein said selective filter is a filter having a pore-size of approximately 10 μ m.
4. A method according to claim 1, wherein trophoblast cells in said separated fraction are specifically identified.
5. A method according to claim 4, wherein the trophoblast cells are identified using a trophoblast-reactive antibody.
6. A method according to claim 5, wherein said antibody is specific for trophoblast cells.
7. A method according to claim 5 or claim 6, wherein said antibody is a monoclonal antibody.
8. A method according to claim 4, wherein the trophoblast cells are identified by *in situ* hybridization.
9. A method according to claim 8, wherein said hybridization is carried out using a probe specific for trophoblast mRNA.

- 25 -

10. A method according to claim 4, wherein the trophoblast cells are identified using both a trophoblast-reactive antibody and a probe specific for trophoblast mRNA.
11. A method for the determination of the level of trophoblast cells in a sample of peripheral blood of a pregnant mammal, which method comprises the steps of (i) separating a fraction comprising trophoblast cells from said peripheral blood sample by selective filtration, and (ii) identifying and quantifying the number of trophoblast cells in said fraction.
12. A method according to claim 11, wherein the pregnant mammal is a pregnant human.
13. A method according to claim 11, wherein said selective filtration is carried out using a filter having a pore-size of approximately 10 μ m.
14. A method according to claim 11, wherein the trophoblast cells in the separated fraction of the peripheral blood sample are identified using a trophoblast-reactive antibody.
15. A method according to claim 14, wherein said antibody is specific for trophoblast cells.
16. A method according to claim 14 or claim 15, wherein said antibody is a monoclonal antibody.
17. A method according to claim 11, wherein the trophoblast cells in the separated fraction of the peripheral blood sample are identified by *in situ* hybridization.

- 26 -

18. A method according to claim 17, wherein said hybridization is carried out using a probe specific for trophoblast mRNA.
19. A method according to claim 11, wherein the trophoblast cells in the separated fraction of the peripheral blood sample are identified using both a trophoblast-reactive antibody and a probe specific for trophoblast mRNA.
20. A method according to claim 12, wherein the quantification of the number of trophoblast cells in the separated fraction of the peripheral blood sample is used to identify individuals who are susceptible to, or predisposed to pregnancy induced hypertension (PIH).
21. A method for obtaining fetal genetic and/or biochemical information from trophoblast cells, which method comprises the steps of (i) separating a fraction comprising trophoblast cells from a sample of peripheral blood of a pregnant mammal by selective filtration, and (ii) obtaining genetic and/or biochemical information from trophoblast cells in said fraction.
22. A method according to claim 21, wherein the pregnant mammal is a pregnant human.
23. A method according to claim 21, wherein said selective filtration is carried out using a filter having a pore-size of approximately 10 μ m.
24. A method according to claim 21, wherein said genetic and/or biochemical information is obtained by *in situ* hybridization using at least one probe specific for chromosomes in the nucleus of trophoblast cells.

- 27 -

25. A kit for separation of trophoblast cells from a sample of peripheral blood of a pregnant mammal, which comprises a selective filter which separates a fraction comprising trophoblast cells from said peripheral blood sample.
26. A kit according to claim 25, wherein said selective filter is a filter having a pore-size of approximately 10µm.
27. A kit for the determination of the level of trophoblast cells in a sample of peripheral blood of a pregnant mammal, comprising (i) a selective filter which separates a fraction comprising trophoblast cells from said peripheral blood sample, and (ii) means for identifying and quantifying the number of trophoblast cells in said fraction.
28. A kit according to claim 27, wherein said selective filter is a filter having a pore-size of approximately 10µm.
29. A kit according to claim 27, comprising a trophoblast-reactive antibody to specifically identify trophoblast cells.
30. A kit according to claim 29, wherein said antibody is a monoclonal antibody.
31. A kit according to claim 27, comprising a probe specific for trophoblast mRNA to specifically identify trophoblast cells.
32. A kit according to claim 27, comprising both a trophoblast-reactive antibody and a probe specific for trophoblast mRNA to specifically identify trophoblast cells.
33. A kit for obtaining fetal genetic and/or biochemical information from trophoblast cells, comprising (i) a selective filter which separates a fraction

- 28 -

comprising trophoblast cells from a sample of peripheral blood of a pregnant mammal, and (ii) means for obtaining genetic and/or biochemical information from trophoblast cells in said fraction.

34. A kit according to claim 33, wherein said selective filter is a filter having a pore-size of approximately 10 μ m.
35. A kit according to claim 33, further comprising means for identifying trophoblast cells in said fraction.
36. A kit according to claim 35, comprising a trophoblast-reactive antibody to specifically identify trophoblast cells.
37. A kit according to claim 36, wherein said antibody is a monoclonal antibody.
38. A kit according to claim 35, comprising a probe specific for trophoblast mRNA to specifically identify trophoblast cells.
39. A kit according to claim 35, comprising both a trophoblast-reactive antibody and a probe specific for trophoblast mRNA to specifically identify trophoblast cells.
40. A kit according to claim 33, comprising at least one probe specific for chromosomes in the nucleus of trophoblast cells to obtain said genetic and/or biochemical information by *in situ* hybridization.



INTERNATIONAL SEARCH REPORT

International application No
PCT/AU 98/00774

A. CLASSIFICATION OF SUBJECT MATTER		
Int Cl ⁶ G01N 33/49, 33/48, 33/57; C12Q 1/68		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) IPC: G01N 33/49, 33/48; C12Q 1/68		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched AU: IPC as above		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WPAT trophoblast or placenta or fetal: and blood JAPIO trophoblast or placenta or fetal: and blood MEDLINE trophoblast or placenta or fetal: and blood		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5646004 A (Van VLASSELAER) 8 July 1997 whole document	1-40
A	US 5641628 A (BLANCHI) 24 June 1997 whole document	1-40
A	US 5503981 A (MUELLER et al) 2 April 1996 whole document	1-40
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C		<input checked="" type="checkbox"/> See patent family annex
<ul style="list-style-type: none"> * Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed 		
Date of the actual completion of the international search 22 October 1998		Date of mailing of the international search report 30.10.98
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929		Authorized officer S CHEW Telephone No.: (02) 6283 2248

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU 98/00774

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5437987 A (TENS et al) 1 August 1995 whole document	1-40
A	WO 90/06509 A (THE FLINDERS UNIVERSITY OF SOUTH AUSTRALIA) 14 June 1990 whole document	1-40

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No
PCT/AU 98/00774

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
US 5646004	AU 35025/95	CA 2198607	EP 778944				
	WO 96/07097	US 5663051	US 5474687				
	US 5789148	US 5648223	AU 14150/97				
	WO 97/21488						
US 5641628	AU 67505/90	AU 24870/95	AU 68026/98				
	EP 500727	EP 791659	WO 91/07660				
US 5503981	AU 46466/89	CA 2004592	CN 1043788				
	DK 1073/91	EP 447424	NO 912133				
	NZ 231621	WO 90/06509					
US 5437987	US 5275933	WO 94/07364					
WO 90/06509	AU 46466/89	CA 2004592	CN 1043788				
	DK 1073/91	EP 447424	NO 912133				
	NZ 231621	US 5503981					
END OF ANNEX							